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# Stress Responses and Sexing of Wild Kemp's Ridley Sea Turtles (*Lepidochelys kempii*) in the Northeastern Gulf of Mexico

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Plasma corticosterone, glucose, and testosterone concentrations were measured in wild, immature specimens of the highly endangered Kemp's ridley sea turtle (*Lepidochelys kempii*) to determine effects of acute handling stress. Thirty-nine free-ranging turtles were captured by entanglement net near the Cedar Keys, Florida. Blood samples were collected immediately after retrieval from the net, and at 30 min ($n$ = 15) and at 60 min ($n$ = 29) thereafter. Mean plasma corticosterone and glucose concentrations increased significantly with time. No significant difference was observed over time for mean testosterone concentrations. Approximately half of the turtles demonstrated an increase in plasma testosterone after 60 min of captivity while the others demonstrated a decrease. Initial testosterone concentrations were used to determine the sex of individual turtles. Fifty-nine percent of turtles were classified as female, 33% as male, and 8% as indeterminant. The results of this study demonstrate a responsive hypothalamic-pituitary-adrenal axis and hyperglycemia in immature Kemp's ridley turtles during acute handling stress 

## INTRODUCTION

Endocrine products of the vertebrate hypothalamic–pituitary–adrenal (HPA) axis have been used extensively as indicators of physiological stress (Stephens, 1980; Axelrod and Reisine, 1984; Greenberg and Wingfield, 1987). Stress-induced elevations of the adrenocortical steroid corticosterone have been demonstrated in a variety of reptiles (Elsey *et al.*, 1990; Mahapatra *et al.*, 1991; Moore *et al.*, 1991; Grassman and Hess, 1992; Lance, 1994). Recent efforts have shown that the HPA axis of wild, immature loggerhead (*Caretta caretta*; Gregory *et al.*, 1996) and green sea turtles (*Chelonia mydas*; Aguirre *et al.*, 1995) is sensitive to stress as indicated by elevated plasma corticosterone concentrations. Effects of stress on the HPA axis have also been reported for Kemp's ridley turtles (*Lepidochelys kempii*, Varlverde *et al.*, 1996), although actual corticosterone values were not given.

It is well established that hyperglycemia occurs in mammals under conditions of stress (Mizock, 1995). Stress enhances catecholamine secretion from the adrenal medulla and sympathetic nerve endings. Epinephrine in particular raises plasma glucose levels by stimulating hepatic glycogenolysis and gluconeogenesis and by interfering with peripheral tissue glucose transport (Yamada *et al.*, 1993). Stress-induced hyperglycemia is largely uninvestigated in reptiles. One published study (Aguirre *et al.*, 1995) demonstrated increased plasma glucose concentrations in response to acute handling stress in wild, juvenile green turtles.

Acute handling stress has been associated with suppression of plasma testosterone (T) concentrations in

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adult, male reptiles. For example, tree lizards (*Urosaurus ornatus*; Moore *et al.*, 1991) and alligators (*Alligator mississippiensis*; Lance and Elsey, 1986) exhibited significant reductions in plasma testosterone concentrations after 4 h of captivity. However, immature loggerhead (Wibbels *et al.*, 1987) and Kemp's ridley (Owens, 1997) turtles have demonstrated both elevations and reductions of plasma testosterone after capture.

Immature sea turtles are sexually monomorphic, and sex identification based on external morphometrics is not possible. An accurate, noninvasive sexing technique is highly desirable for sea turtle conservation and management purposes. Since the 1970s, relatively high plasma T concentrations have been used to determine the sex of juvenile sea turtles (Owens *et al.*, 1978; Wibbels *et al.*, 1987). Recently, sexing criteria for Kemp's ridley turtles were developed using plasma T concentrations and verified by laparoscopic examination of the gonads (Coyne and Landry, 2000).

Relatively few data exist on stress responses in wild populations of ectothermic vertebrates. The present study investigates the effects of acute handling stress (defined here as the stress of capture, repeated bleeding, and restraint up to 1 h) on plasma concentrations of corticosterone, glucose, and testosterone in wild, immature Kemp's ridley turtles. In addition, initial testosterone concentrations were used for sex determination. To our knowledge, no other published study has reported values for stress-induced elevations of corticosterone or glucose for wild Kemp's ridley turtles. These data provide valuable information for comparative analyses of the vertebrate stress response and will give insight into the endocrinology of the most endangered species of sea turtles.

## MATERIALS AND METHODS

### *Animals*

Entanglement nets were used to capture Kemp's ridley turtles near the Cedar Keys, Florida, from May to November 1992. Detailed descriptions of the study site and capture techniques are provided by Schmid (1998) and Gregory *et al.* (1996). Nets were constantly monitored and turtles were retrieved immediately after surfacing. Initial blood samples, arbitrarily designated Time zero (*T*-0; $n = 39$), were collected within 15 min of sighting the turtles in the net. Samples (3 ml) were collected from the dorsal cervical sinus using 20-gauge needles and sterile, sodium-heparinized vacuum tubes. Additional blood samples were taken at 30 min (*T*-30; $n = 15$) and 60 min (*T*-60; $n = 29$). Only one or two samples were taken from turtles $< 40$ cm minimum straight-line carapace length (MSCL; Pritchard *et al.*, 1983) to protect the smaller animals from the possible negative effects of hemodilution. Some turtles were released prior to *T*-60 sampling because of inclement weather. Turtles were kept turned on their carapace and in the shade between sample collections. Blood samples were centrifuged (1200*g*) for 5 min immediately after collection. Plasma was transferred into cryovials, frozen in liquid nitrogen within 15 min of sampling, and stored at $-70$°C until assayed. After blood sampling, turtles were measured, tagged, and photographed as described by Schmid (1998). Tail measurements were made with a flexible fiberglass measuring tape from the posterior margin of the plastron to tip of the tail (PT) and from the posterior margin of the plastron to midcloaca (PC).

### *Sample Analyses*

Plasma samples from *L. kempi* were analyzed for corticosterone using a standard radioimmunoassay (RIA) similar to one developed for *C. caretta* (Gregory *et al.*, 1996). Antiserum (No. 07-120016, lot 3R3-PB) and tritium-labeled corticosterone were purchased from ICN Biomedicals (Costa Mesa, CA). The minimum detectable limit was 12 pg/ml. Values were determined by a cubic spline model using a program supplied by Beckman Inc. (EIARIA Curve Fit Program). All samples (200 ml) were extracted twice with 4 ml of anhydrous ether prior to RIA analyses (extraction efficiency, 86 ± 6%). Standard curves were prepared with known amounts of radioinert corticosterone (0, 0.31, 0.62, 1.25, 2.50, 5, 10, 20, 40, 80 ng/ml) purchased from Amersham Corporation (Arlington Heights, IL). Final characterization of the assay involved measurement of known amounts of radioinert corticosterone (0, 0.31, 0.62, 1.25, 2.5, 5, 10, 20, 40, 80 ng/ml) in 50 $\mu$l of charcoal-stripped plasma [$Y = 0.615X + 0.980$; $Y$ = amount of corticosterone measured (ng/ml); $X$ = amount of corticosterone added (ng/ml); $R^2 = 0.965$].

Inter- and intra-assay coefficients of variation were 12.4 and 7.5%, respectively.

The testosterone RIA procedure was identical to one developed for *L. olivacea* (Valverde, 1996). Inter- and intra-assay coefficients of variation were 8.5 and 6.5%, respectively. Coyne and Landry (2000) developed sexing criteria for *L. kempi* captured by a tangle net at Sabine Pass, Texas using initial T concentrations ($N > 80$ verified by laparoscopy; Coyne, personal communication). Turtles with T concentrations below 12 pg/ml were classified as female and those above 18 pg/ml were classified male. Turtles with T concentrations between these criteria were classified as indeterminant. These criteria were applied to the *T*-0 samples collected in the present study, but sex was not verified by laparoscopic examination.

A Glucose LiquiColor enzymatic-colorimetric test (Procedure No. 1070) from STANBIO Laboratory, Inc. (San Antonio, TX) was used to determine plasma glucose concentrations. Values were determined by a linear model using a program supplied by BIO-RAD Microplate Manager (Hercules, CA). Standard curves were prepared with known amounts of glucose (0, 25, 50, 100, and 200 mg/dl) in distilled water [$Y = 533.333x + 7.989$; $Y$ = amount of glucose measured (mg/dl); $X$ = absorbance; $R^2 = 0.989$]. Plasma samples (0.01 ml) or standards (0.01 ml) were mixed with glucose oxidase reagent (1.0 ml) and incubated at room temperature for 20 min. Sample and standard aliquots (250 ml) were transferred in duplicate to a microplate and their absorbance was determined ($\lambda = 480$ nm). Inter- and intra-assay coefficients of variation were 6.2 and 2.5%, respectively.

### *Statistics*

Statistical analyses were performed using the Super ANOVA general linear modeling program (Abacus Concepts, 1991). Corticosterone, testosterone, and glucose concentrations (ng/ml, pg/ml, and mg/dl, respectively) were log transformed to obtain homogeneity of variance. All reported probability values were obtained from log-transformed data. All reported graphs and mean values (mean ±SE) were obtained from raw data. Statistical significance was accepted at $P < 0.05$.

Data from serial samples were subjected to repeated measures analyses of variance (ANOVA). Mean comparison contrasts were performed and the $P$ value for each contrast was multiplied by the number of comparisons made (Bonferroni; Jandel Scientific, 1994). The effect of number of blood samples on *T*-60 corticosterone concentrations was determined by an analysis of covariance (ANCOVA) with MSCL as the covariate. A $\chi^2$ test was used to detect a significant difference from a predicted sex ratio of 1 F:1 M. Linear regressions were performed among initial hormone concentrations and MSCL to detect any associations.

## RESULTS

Plasma corticosterone concentrations ranged from 0.03 ng/ml (sampled at *T*-0 with MSCL = 44.5 cm) to 82.87 ng/ml (sampled at *T*-60 with MSCL = 32.3 cm). Mean corticosterone concentrations for Kemp's ridley turtle plasma samples increased over time ($P = 0.0001$; Fig. 1A). Values at *T*-30 (13.08 ± 3.53 ng/ml) and *T*-60 (24.68 ± 3.65 ng/ml) were significantly higher ($P = 0.0003$) than mean corticosterone concentrations at *T*-0 (6.16 ± 2.31 ng/ml). Corticosterone concentrations were higher in turtles sampled twice than those sampled three times ($P = 0.0339$; Table 1). Due to our sampling protocol, however, the mean MSCL was significantly higher in turtles sampled three times ($P = 0.0032$; Table 1) and accounted for the variation due to the number of samples.

Glucose levels ranged from 41.30 mg/dl (sampled at *T*-0 with MSCL = 35.1 cm) to 175.10 mg/dl (sampled at *T*-0 with MSCL = 35.1 cm). A gradual but significant increase was also observed for mean glucose concentrations over time ($P = 0.0007$; Fig 1B). The mean value at *T*-60 (106.43 ± 4.82 mg/dl) was significantly higher than that at *T*-0 (94.41 ± 3.78 mg/dl). No significant difference occurred between mean values of plasma glucose at *T*-0 and *T*-30 (101.49 ± 5.89 mg/dl).

Mean testosterone concentrations did not vary significantly over time ($P > 0.68$; Fig. 1C). A high degree of individual variability was observed, with T levels increasing in 55% of the turtles and decreasing 45% by *T*-60. Initial T concentrations produced a sex ratio of 1.8 F:1.0 M (Fig. 2) that was not significantly different from 1 F:1 M ($\chi^2 = 2.78$, $df = 1$, $P = 0.0956$). Furthermore, there was no significant bias in the sex ratios of

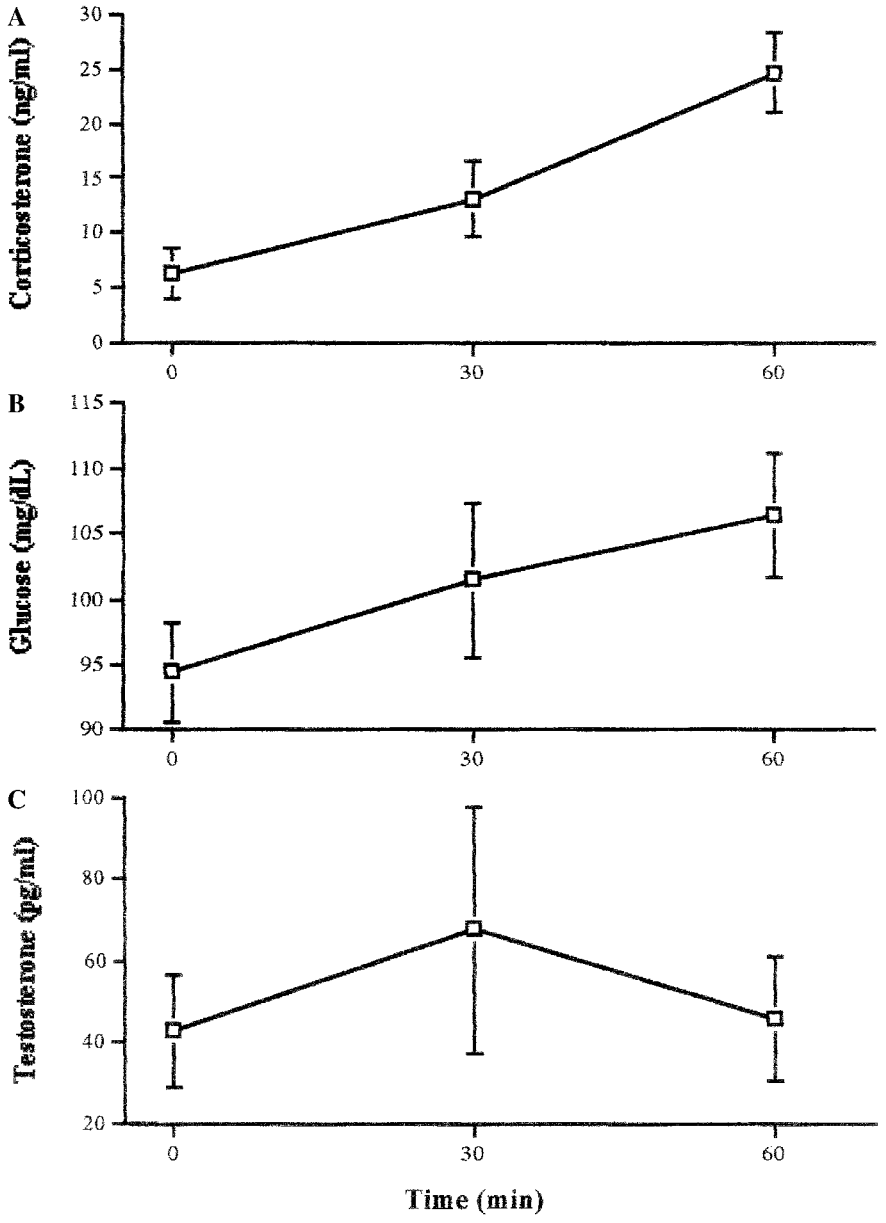


FIG. 1. Mean plasma concentrations of (A) corticosterone, (B) glucose, and (C) testosterone in Kemp's ridley turtles over time. Repeated measures ANOVA resulted in significant elevations of corticosterone and glucose but not testosterone due to high individual variability.

turtles $< 40$ cm MSCL (9 F:3 M, $\chi^2 = 3.00$, $df = 1$, $P = 0.0833$) and $> 40$ cm (14 F:10 M, $\chi^2 = 0.67$, $df = 1$, $P = 0.4142$). The changes in T concentration over time did not reclassify the sex of turtles at *T*-60. However, two indeterminants could be reclassifed as females when using T values at *T*-30.

T concentrations for predicted males ranged from 20.6 pg/ml (sampled at *T*-0, with MSCL = 35.1 cm) to 427.0 pg/ml (sampled at *T*-0 with MSCL = 43.7 cm) and for predicted females ranged from 2.0 pg/ml (sampled at *T*-30 with MSCL = 41.1 cm) to 10.5 pg/ml (sampled at *T*-0 with MSCL = 50.8 cm). Males with carapace lengths of 37–45 cm exhibited relatively high T levels (84–427 pg/ml; Fig. 2). Predicted females exhibited increasing T concentrations with increasing carapace length ($P = 0.0345$).

Mean MSCL of females (42.7 ± 1.4 cm) did not differ significantly from mean MSCL of males (42.3 ± 1.7

TABLE 1
Mean plasma corticosterone concentrations at 60 min and carapace length for Kemp's ridley turtles sampled 2 or 3 times.

| Number of Bleedings | *n* | Mean corticosterone concentration at T-60 | Mean carapace length |
|---|---|---|---|
| 2 | 14 | 31.24 ng / mL (± 5.50) | 39.7 cm (± 1.9) |
| 3 | 15 | 18.55 ng / mL (± 4.45) | 47.0 cm (± 1.3) |

cm). There was no significant difference ($t = 1.49$, $P = 0.0722$) in PT length between predicted males (9.73 ± 0.29 cm) and females (8.65 ± 0.46). However, mean PC length for predicted males (6.39 ± 0.37 cm) was significantly larger ($t = 1.81$, $P = 0.0394$) than that for predicted females (5.52 ± 0.29 cm).

During the present study, the water temperature increased from 27°C in May to 30°C in June through August, peaked at 31°C in early September, and decreased to 24°C by the end of October. No significant correlations were observed between month and initial corticosterone, glucose, or T concentrations. Furthermore, no significant correlations were detected among corticosterone, glucose, T, and MSCL.

## DISCUSSION

This study demonstrates that the HPA axis of wild, immature Kemp's ridley turtles is sensitive to acute handling stress, as indicated by significant increases of plasma corticosterone concentrations over time. Twenty-seven of 29 animals experienced elevations of corticosterone, with several individuals exhibiting increases of over 15 times higher than initial levels after 60 min of captivity. Stress-induced elevations of corticosterone have been observed in wild immature and adult loggerhead (Gregory *et al.*, 1996) and immature green (Aguirre *et al.*, 1995) and adult (*Lepidochelys olivacea*; Valverde, 1996) olive ridley sea turtles. Initial mean corticosterone concentrations of Kemp's ridley turtles in the present study were at least 8-fold higher than these other species. Two turtles had initial values over 3 SD from the mean (43.08 and 80.85 ng / ml). We speculate that these individuals may have been stressed before the initial blood sample, especially since the animal with the highest level was the only turtle to exhibit a decrease in corticosterone over time. Excluding these two initial values resulted in a lower initial mean concentration of 3.14 ± 0.68 ng / ml, which may be more indicative of baseline for wild, immature Kemp's ridley sea turtles. Nevertheless, an interspecific difference is observed as this value is 5.7-fold higher than the mean initial corticosterone value of wild, immature loggerheads collected by the same capture technique, during the same year, and in the same location (Gregory *et al.*, 1996).

Kemp's ridley and loggerhead turtles exhibited behavioral differences during the study. Kemp's ridley turtles were more active, often demonstrating bursts of flipper movements while turned and attempting to bite researchers when handled. These behaviors were observed to a much lesser degree in loggerhead turtles. Several green turtles captured during the course of the study were even more subdued. These interspecies differences in behavior are noted in a classic publication by Carr (1942): "While captured loggerheads and green turtles may be handled with comparative (the latter with complete) impunity, the ridley exhibits almost hysterical violence and obstinacy when caught." Glucocorticoids are thought to influence behavior in several species of reptiles, although studies focus on adult males (Schuett *et al.*, 1996; Knapp and Moore, 1997). It is possible that the relatively high mean, initial corticosterone value observed in Kemp's ridley turtles may have some functional significance and perhaps is associated with this species higher level of aggression, but correlations between glucocor-

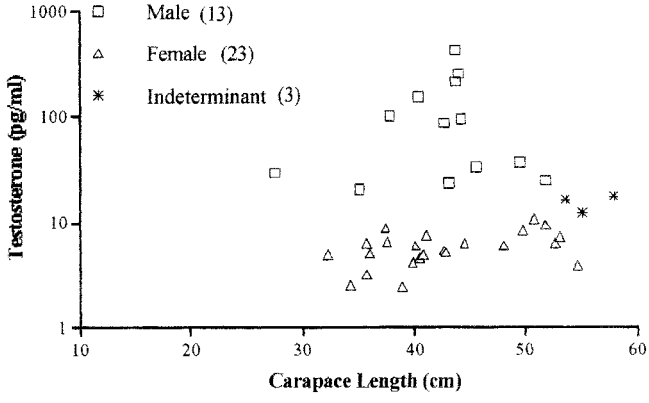


FIG. 2. Initial plasma testosterone concentrations for immature Kemp's ridley turtles showing predicted males and females. The *y* axis was log transformed to better visualize the data. Numbers in parentheses indicate sample size.

ticoid levels and behavior among sea turtle species are untested. Additionally, the elevated values observed for Kemp's ridley turtles might make this species more susceptible to stress-induced mortality, such as incidental capture in commercial fisheries.

It was expected that Kemp's ridley turtles sampled three times would experience higher corticosterone levels than those sampled twice due to repeated handling and possible hemodilution. However, corticosterone concentrations were significantly higher in smaller turtles sampled twice. These data indicate a size effect on stress-induced elevations of corticosterone in immature Kemp's ridley turtles. Whether this effect of size is due to higher metabolic rates of smaller animals or some other aspect of the HPA response is not known. One published study demonstrated higher corticosterone levels in small versus large loggerhead sea turtles, but the variation was thought to be associated with reproductive condition and not metabolic rates (Gregory *et al.*, 1996). Further studies of size effects on stress-induced elevations of corticosterone in reptiles are required to clarify these data.

This is the first study to demonstrate a hyperglycemic response to acute handling stress in wild, immature Kemp's ridley turtles. Stress-induced hyperglycemia has been observed in immature green turtles (Aguirre *et al.*, 1995) and mean glucose values from the present study were comparable to those observed for immature green turtles. However, mean initial glucose levels for nesting Olive ridley turtles were over 30 mg/dl lower (Valverde, 1996) than those reported presently for Kemp's ridley turtles. In addition, a decrease in plasma glucose concentrations was observed in nesting Olive ridley turtles after 1 h of turning stress (Valverde, 1996). Thus, it would seem that reproduction and size class may be factors affecting stress-induced responses of glucose in sea turtles.

The HPG axis seems to respond in a highly complex manner as no general trend was observed in the response of plasma T concentrations to handling stress (i.e., T concentrations increased over time in some predicted males and females and decreased in others). Owens (1997), citing unpublished work of Coyne and Landry, indicated that initial blood samples predict sex well in immature Kemp's ridley turtles, but subsequent samples collected over several hours showed increased variability. He suggested that handling stress alters plasma T concentrations. The results of the present study also demonstrated individual alterations of plasma T concentrations, presumably associated with acute stress-induced elevations of corticosterone. Most studies demonstrating that corticosterone and T are negatively correlated focus on effects of chronic stress on adult male reptiles. We propose that maturity status and the duration of exposure to stress may be factors in determining whether corticosterone will have suppressive effects on plasma T during stress. In addition, studies show that reptilian adrenal glands can secrete significant levels of T (Manzo *et al.*, 1994). Often, it is assumed that the plasma T being measured is of gonadal origin. This assumption may not be applicable to immature reptiles.

Since the 1970s, plasma T concentrations have been used to distinguish immature male from female sea turtles. However, care should be taken when utilizing absolute values of plasma hormones to predict sex. Studies have indicated that plasma T concentrations can be highly variable and thus questionable in sexing turtles (Schroeder and Owens, 1994; Gregory, 1994; Braun-McNeill *et al.*, 2000). The T values used to distinguish male from female also differ within species at different research sites (reviewed in Owens, 1997). This would indicate a location effect and would require validation of sex by laparoscopy every time a new research site is utilized. In addition, a very small range of T values (e.g., $< 10$ pg/ml) separates many of the male–female sexing criteria. Any slight variation in physiological levels of T or radioimmunoassay procedures could lead to false results. A ratio of estradiol to T (E/T) was found to be more accurate at predicting sex in hatchling loggerhead turtles than either hormone alone (Gross *et al.*, 1995). However, E/T ratios need to be validated for different size classes and species before determining whether it is more accurate than T concentrations alone.

Sex ratio is a vital component for modeling the demographics of endangered species and information has been accumulating on the sex of immature Kemp's ridley turtles in the wild (Owens 1997). Danton and Prescott (1988) reported a sex ratio of 1.4 F:1 M for juvenile Kemp's ridley turtles stranded in Cape Cod Bay, Massachusetts. Morreale *et al.* (1992) noted a 2 F:1 M ratio for cold-stunned turtles in Long Island Sound, New York. Strandings of wild Kemp's ridleys in Texas have yielded ratios of 1 F:1.8 M on the lower coast (Shaver, 1991) and 3 F:1 M on the upper coast (Stabenau *et al.*, 1996). Coyne and Landry (2000) used

laparoscopy and plasma T to sex wild turtles ($n = 179$) captured in the northwestern Gulf of Mexico and detected no significant bias in sex ratio (1.1 F:1.0 M). Comparably, the results of our RIA analysis in the northeastern Gulf found no significant departure from a ratio of 1 F:1 M, although there were almost twice as many predicted females as predicted males. Larger sample sizes are required for a more accurate assessment of sex ratio in the latter population of Kemp's ridley.

The relatively higher initial T levels observed in male Kemp's ridley turtles with MSCL > 37 cm suggest that the testis may be maturing at this size class. Coyne and Landry (2000) observed a similar pattern in their sample of wild Kemp's ridley turtles. The significant positive correlation between testostorone and female carapace length in the present study may also indicate maturation of ovaries. Carr and Caldwell (1956) observed follicles the size of "b-b shot" in Kemp's ridley females as small as 50 cm converted MSCL (Schmid, 1998). Coyne and Landry (2000) suggested redefining Ogren's (1989) developmental stages in terms of the physiological data. Owens (1997) equates the subadult stage of marine turtle development with the period of pubertal changes. If indeed these hormone data reflect gonadal maturation, we concur with these authors and suggest the following modifications to Ogren's (1989) size classes: pelagic juvenile (5–19 cm), coastal-benthic juvenile (20–39 cm), coastal-benthic subadult (40–59 cm), and coastal-benthic adult (> 60 cm).

Tail length is the major secondary sexual characteristic for mature sea turtles. The adult male has a longer, thicker tail with a more distally located cloaca, but the relationship of tail length to sex is not known for immature turtles (Pritchard *et al.*, 1983). The Kemp's ridley turtles of the present study did not express the dimorphic characteristics of total tail length, but predicted males did exhibit a significantly greater distance between the plastron and cloaca (more distally located). While this subtle difference between predicted sexes is not practical for sexing immature turtles, it does suggest that morphological differentiation between sexes may begin prior to maturation.

In conclusion, the results of our study show that immature Kemp's ridley turtles respond to handling stress in terms of elevated plasma corticosterone and hyperglycemia. In addition, an effect of size on stress-induced elevations of corticosterone was indicated, with smaller turtles having higher corticosterone concentrations after 30 min of capture. Plasma corticosterone and glucose concentrations might be used to assess the health of other aggregations of Kemp's ridley turtles provided our initial samples are representative of baseline values. The physiological function of corticosterone is still unclear in reptiles. A 0.47-kb fragment of the glucocorticoid receptor (GR) from alligator adrenal gland has been cloned and sequenced (Gregory, Blumberg, and Lance, unpublished data) in order to design a molecular marker for alligator GR mRNA. Studies utilizing such molecular tools are required to begin to assess the function of this stress hormone.

T concentrations indicated that Kemp's ridley turtles may begin maturing at 40 cm carapace length, but studies are needed to verify whether plasma T can be used to indicate gonadal maturation. Stress-induced alterations of plasma T were observed and must be avoided when using T concentrations for sexing purposes. Hormone values should be interpreted with caution as interassay variability alone can lead to false results (Gregory, 1996), especially when a small number of pg/ml separate male from female. Development of new sexing technology is desirable (reviewed in Wibbels *et al.*, 2000). Researchers often analyze reptilian plasma for the types of hormones found predominately in mammalian plasma. However, other sex steroids (e.g., deoxytestosterone, estrone) may be more accurate indicators of sex in reptiles.

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